Molecular Biology of the Cell
Vol. 17, 944–954, February 2006

Cell Cycle-dependent Recruitment of Telomerase RNA and Cajal Bodies to Human Telomeres

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Submitted September 30, 2005; Revised November 4, 2005; Accepted November 17, 2005
Monitoring Editor: Marvin P. Wickens

Telomerase is a ribonucleoprotein enzyme that counteracts replicative telomere erosion by adding telomeric sequence repeats onto chromosome ends. Despite its well-established role in telomere synthesis, telomerase has not yet been detected at telomeres. The RNA component of human telomerase (hTR) resides in the nucleoplasmic Cajal bodies (CBs) of interphase cancer cells. Here, in situ hybridization demonstrates that in human HeLa and Hep2 S phase cells, besides accumulating in CBs, hTR specifically concentrates at a few telomeres that also accumulate the TRF1 and TRF2 telomere marker proteins. Surprisingly, telomeres accumulating hTR exhibit a great accessibility for in situ oligonucleotide hybridization without chromatin denaturation, suggesting that they represent a structurally distinct, minor subset of HeLa telomeres. Moreover, we demonstrate that more than 25% of telomeres accumulating hTR colocalize with CBs. Time-lapse fluorescence microscopy demonstrates that CBs moving in the nucleoplasm of S phase cells transiently associate for 10–40 min with telomeres. Our data raise the intriguing possibility that CBs may deliver hTR to telomeres and/or may function in other aspects of telomere maintenance.

INTRODUCTION

Telomeres are specific nucleoprotein complexes protecting the termini of eukaryotic linear chromosomes from degradation, end-to-end fusion and undesired recombination (for recent reviews, see Wong and Collins, 2003; Ferreira *et al.*, 2004; Smogorzewska and de Lange, 2004; Blasco, 2005). In humans, the DNA component of telomere is made up of ~5–15 kb of double-stranded 5′-TTAGGG-3′ repeats that terminates in a 3′ overhang of ~50–300 bases (Makarov *et al.*, 1997; Wright *et al.*, 1997). By invading into proximal double-stranded telomeric sequences, the single-stranded overhang supports formation of a large duplex loop, termed the T-loop (Griffith *et al.*, 1999). Because of the unidirectionality of conventional DNA polymerases, the ends of telomeres cannot be fully duplicated and human telomeres lose ~50–200 base pairs during each cell division cycle and eventually, critically short telomeres induce cell cycle arrest called proliferative senescence. Therefore, by imposing a limit on the replicative life span of somatic cells, telomere erosion represents an innate mechanism for tumor suppression (Smogorzewska and de Lange, 2004).

The replicative telomere erosion can be balanced by the telomerase reverse transcriptase that adds telomeric DNA repeats to the 3′ overhang of telomeres (reviewed in Collins and Mitchell, 2002; Cong *et al.*, 2002). In humans, telomerase activity is not detectable in most somatic cells, but germ line and other highly proliferative cells as well as the majority of tumor-derived cell lines possess highly active telomerase, indicating that maintenance of telomere length is necessary for indefinite proliferation of human cells (Kim *et al.*, 1994; Greider, 1996; Shay and Bacchetti, 1997). Telomerase is a ribonucleoprotein (RNP) enzyme that is minimally composed of the telomerase RNA (TR) that specifies the repeat sequence added and the telomerase reverse transcriptase (TERT) protein (Greider and Blackburn, 1989; Weinrich *et al.*, 1997; Beattie *et al.*, 1998). The human TR (hTR) is a 451-nt-long RNA that is composed of two major structural domains. The 5′-terminal template domain of hTR folds into an evolutionarily conserved pseudoknot structure and the 3′-end region possesses a "hairpin-hinge-hairpin-tail" secondary structure that is indistinguishable from the consensus structure of box H/ACA pseudouridylation guide RNAs (Mitchell *et al.*, 1999; Chen *et al.*, 2000). Although no evidence suggests that hTR could function in RNA pseudouridylation, the mature telomerase RNP is associated with the complete set of box H/ACA RNP proteins, including the pseudouridine synthase dyskerin (Meier, 2005).

The 3′-terminal box H/ACA domain provides metabolic stability for hTR and it is also essential for telomerase function (Mitchell *et al.*, 1999; Mitchell and Collins, 2000; Martin-Rivera and Blasco, 2001; Chen *et al.*, 2002; Fu and Collins, 2003). Moreover, the box H/ACA domain determines the correct intracellular localization of hTR (Jády *et al.*, 2004). The apical loop of the 3′-terminal hairpin of hTR carries a Cajal body (CB) localization signal, the CAB box, that is also present in a subclass of box H/ACA pseudouridylation guide RNAs that accumulate in CBs and are called small CB-specific RNAs (scaRNAs; Richard *et al.*, 2003). Indeed, hTR specifically localizes to CBs in a CAB box-dependent manner, indicating that hTR can be considered as a scaRNA (Jády *et al.*, 2004; Zhu *et al.*, 2004).

This article was published online ahead of print in *MBC in Press* (http://www.molbiolcell.org/cgi/doi/10.1091/mbc.E05–09–0904) on November 30, 2005.

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Abbreviations used: CB, Cajal body; hTR, human telomerase RNA; hTERT, human telomerase reverse transcriptase.

The functional importance of accumulation of hTR in CBs remains unknown. CBs are evolutionarily conserved subnuclear compartments that vary both in size (0.2–1.5 μm in diameter) and number (0–10), depending on the cell type (reviewed in Ogg and Lamond, 2002; Gall, 2003; Cioce and Lamond, 2005). CBs are most prominent in cells that show high transcriptional activity and are actively dividing. Several lines of evidence indicate that at least one function of CBs is in the assembly and maturation of small nuclear and nucleolar RNPs (snRNPs and snoRNPs) and other protein machines involved in mRNA and rRNA biogenesis. It is therefore possible that CBs may also provide the nuclear locale for the assembly and/or maturation of telomerase holoenzyme (Jády *et al.*, 2004; Zhu *et al.*, 2004). However, in contrast to snRNPs and snoRNPs which transiently appear in CBs before accumulating in the nucleoplasm or in the nucleolus where they function, hTR appears to permanently reside in CBs. This raises the intriguing possibility that sequestering of hTR into CBs may play a regulatory role in telomere elongation and/or CBs may directly participate in telomere biogenesis (Jády *et al.*, 2004). Studies in living plant and mammalian cells revealed that CBs are mobile organelles (Boudonck *et al.*, 1999; Platani *et al.*, 2000, 2002; Snaar *et al.*, 2000; Gorisch *et al.*, 2004). Movement of CBs within the interchromatin space is often interrupted by transient interactions with chromatin (Platani *et al.*, 2002). In fact, earlier studies frequently found CBs to be associated with small nuclear RNA (snRNA) and histone genes, leading to the speculation that CBs may act as nucleoplasmic transport or sorting structures (reviewed in Matera, 1999; Ogg and Lamond, 2002).

In this study, by taking advantage of the oligonucleotide fluorescence in situ hybridization (FISH) procedure established earlier to visualize hTR (Jády *et al.*, 2004), we systematically analyzed the intranuclear localization of hTR in synchronized human HeLa and Hep2 cancer cells. We demonstrate that during S phase, hTR specifically accumulates at a few telomeres that frequently colocalize with CBs. By using in vivo time-lapse microscopy, we also demonstrate that in S phase cells, CBs transiently associate with telomeres.

MATERIALS AND METHODS

Plasmid Construction and Cell Cultures

To generate the pGFP-TRF1 expression plasmid, the coding region of the human TRF1 gene was PCR-amplified using the pBluescriptII-HA-TRF1 plasmid (Kim *et al.*, 1999; kind gift of Dr. J. Campisi) as a template and the obtained PCR fragment was inserted downstream to the GFP gene controlled by the human L30 promoter by using the GATEWAY (Invitrogen, Carlsbad, CA) recombination system. To obtain pGFP-TRF2 and pHcRed-TRF2, the *Eco*RI-*Hin*dIII fragment of pAD-hTRF2 (Karlseder *et al.*, 1999; provided by Dr. T. de Lange) carrying the coding region of TRF2 was inserted into the same sites of the pEGFP-C1 and pHcRed-C1 vectors (BD Biosciences Clontech, San Jose, CA). Construction of pDsRFP-coilin has been described (Boulon *et al.*, 2002). HeLa and Hep2 cells were grown in DMEM supplemented with 10% fetal calf serum (FCS; Invitrogen). Transfection was performed with FuGENE 6 (Roche, Indianapolis, IN) transfection reagent according to the manufacturer's instructions. HeLa cells were synchronized by double thymidine blocking (Galavazi *et al.*, 1966). FACS analysis was performed as reported (Crissman and Steinkamp, 1973) and the results were analyzed with CELLQuest software (Becton Dickinson, Lincoln Park, NJ).

Fluorescence Microscopy of Fixed Cells and Signal Quantification

FISH with oligonucleotide probes has been described (http://singerlab.aecom.yu.edu). Oligonucleotides containing aminoallyl-T nucleotides were labeled with FluoroLink Cy3 or Cy5 monofunctional reactive dye (Amersham Biosciences, Piscataway, NJ) and were used as probes to detect hTR (Jády *et al.*, 2004) and telomeric DNA (5′-CT*AACCCTAACCCT*AACCCTAACCCT*A-3′, aminoallyl-modified thymidines are marked by asterisks). Hybridization reactions were carried out in 40% formamide/2× SSC at 37°C. Human coilin was detected with a polyclonal rabbit anti-coilin antibody (1:400 dilution; provided by Dr. A. Lamond) followed by incubation with an anti-rabbit antibody-FITC conjugate (1:300 dilution; Sigma-Aldrich, St. Louis, MO). SMN, RPA14, RPA34, and PCNA proteins were detected by monoclonal mouse anti-SMN (1:500 dilution; BD Biosciences), anti-RPA14 (1:50 dilution, Abcam, Cambridge, United Kingdom; ab6432), anti-RPA32 (1:100 dilution, Interchim, Montluçon, France; clone 9H8) and anti-PCNA (1:900 dilution, Abcam, ab29) antibodies in combination with an anti-mouse-FITC conjugate (1:100 dilution; Jackson ImmunoResearch Laboratories, West Grove, PA). Slides were mounted in mounting media containing 90% glycerol, 1× PBS, 0.1 μg/ml DAPI, and 1 mg/ml *p*-phenylenediamine. Images were acquired at RT on a DMRA microscope (Leica, Deerfield, IL) equipped for epifluorescence, with Leica PL APO lenses (100×/1.40–0.7) and with a CoolSNAP camera (Photometrics, Tucson, AZ) controlled by MetaMorph software (Universal Imaging, West Chester, PA). Images were pseudocolored with Adobe Photoshop (San Jose, CA).

To quantify the light emitted by fluorescent oligonucleotides hybridized to telomeres, digital images from a series of eight focal planes were acquired at 400-nm *z*-intervals. Images were restored by the iterative deconvolution algorithm of the Huygens Professional Software (Scientific Volume Imaging BV, Hilversrum, The Netherlands). To determine the total fluorescence intensity detected at each telomere, the restored images were analyzed with Imaris Software (Bitplane AG, Zurich, Switzerland). The amount of light per telomere was calibrated by measuring the total fluorescence intensity of known number of probe molecules (Femino *et al.*, 1998). Probe molecules were dispersed in 5 μl of mounting medium with final concentrations ranging from 0.25 to 4 ng/μl. Probe solutions were distributed between a slide and a coverslip of known area. For each solution, several single plane images were taken under identical conditions as for the hybridized cells. By using the MetaMorph software (Universal Imaging), the average gray level/pixel was determined and plotted on a graph against concentration. The resulted calibration curve was used to define the number of probe molecules detected at telomeres.

Microscopy of Living Cells

HeLa cells were grown on coverslips with a diameter of 40 mm and were synchronized with double thymidine blocking. One hour before the second thymidine block, cells were transfected with a mixture of pGFP-TRF2 and pRFP-coilin expression plasmids. One hour after the release from the second block, the cells were mounted in DMEM/10% FCS (without phenol red and riboflavin) by using a closed, heated chamber (FCS2, Bioptechs, Butler, PA). Image acquisition conditions were identical to that applied for fixed cell microscopy, except that the temperature of both the chamber and the objective was 37°C. Images from a series of four or five focal planes at 400-nm *z*-intervals were obtained every 3 or 4 min over a time period of 1–2 h. Images were recorded using a binning of 2 × 2. Images were restored by the iterative deconvolution algorithm of the Huygens Professional Software (Scientific Volume Imaging BV). The restored images were analyzed and pseudocolored with Imaris Software (Bitplane AG).

RESULTS

Detection of Novel Nucleoplasmic Foci Accumulating hTR

hTR is a CB-specific RNA that shares a common CB localization signal with box H/ACA pseudouridylation guide scaRNAs (Jády *et al.*, 2004; Zhu *et al.*, 2004). However, although box H/ACA scaRNAs direct pseudouridylation of spliceosomal snRNAs within CBs (Jády *et al.*, 2003), there are no data yet to indicate that hTR could function in this nuclear compartment. This suggests that at some stage of the cell cycle, hTR likely leaves CBs to accumulate at telomeres. To test this hypothesis, human HeLa cells were synchronized by double thymidine blocking that arrests cells in the G1 to S phase transition (Galavazi *et al.*, 1966). Progression of synchronized cells through the cell cycle was monitored by FACS analysis (Figure 1). In G1, S, and G2 phase cells, the intracellular distribution of hTR was investigated by FISH using a mixture of three fluorescently labeled oligonucleotide probes complementary to different regions of hTR (Jády *et al.*, 2004). CBs were visualized by staining the cells with antibodies directed against coilin or the survival of motor neurons (SMN) protein, two marker proteins of the CB (Andrade *et al.*, 1991; Liu and Dreyfuss, 1996; Matera, 1999; Cioce and Lamond, 2005). As reported before (Jády *et al.*, 2004), hTR colocalized with coilin and SMN in CBs during

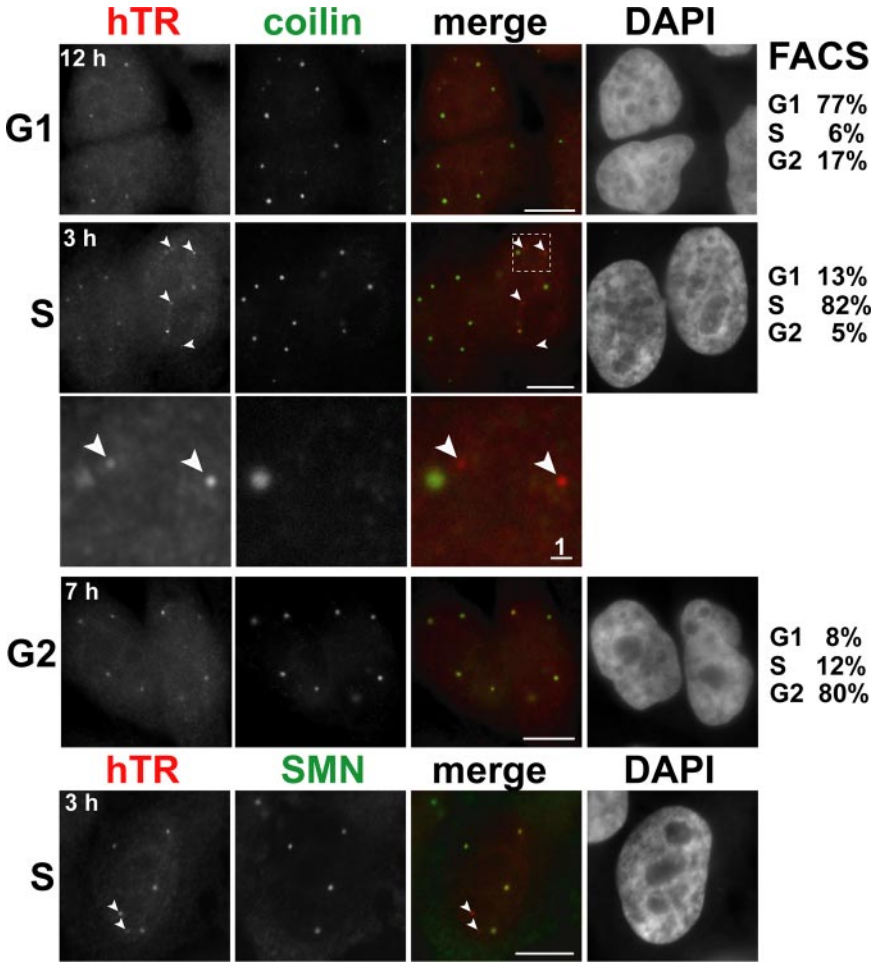

Figure 1. In situ localization of hTR in synchronized HeLa cells. HeLa cells were synchronized and the results of FACS analysis are indicated on the right. At 3 (S phase), 7 (G2 phase), and 12 (G1 phase) h after being released from the second thymidine block, cells were fixed and double-stained with a mixture of hTR-specific fluorescent oligonucleotides (red) and antibodies against coilin or SMN (green). Nuclei were visualized by DAPI staining. White arrowheads indicate nucleoplasmic foci accumulating hTR but lacking coilin and SMN. An enhanced magnification of a nucleoplasmic area (boxed by dashed line) is shown. Unless indicated otherwise, bars, 10 μm.

the whole cell cycle (Figure 1 and unpublished data). However, closer inspection of the double-stained S phase cells revealed that besides concentrating in CBs, hTR frequently accumulated at a few sharp dotlike nucleoplasmic foci (white arrowheads). The newly detected hTR-specific foci were significantly smaller than CBs (~0.1–0.4 μm in diameter; see magnified images) and contained neither coilin nor SMN, indicating that they are not related to the CB. The latter conclusion was corroborated by the fact that the U85 canonical scaRNA failed to localize to the new hTR-specific foci (unpublished data). We therefore concluded that in HeLa S phase cells, besides accumulating in CBs, hTR can also localize to small nucleoplasmic foci of unknown nature.

hTR Accumulates at Telomeres in HeLa S Phase Cells

The biological significance of the newly detected hTR-containing nuclear foci was strongly supported by the fact that they were observed only in S phase cells (Figure 1 and see below). Because telomere synthesis occurs during S phase (Ten Hagen *et al.*, 1990; Wright *et al.*, 1999; Marcand *et al.*, 2000), we reasoned that the new hTR-specific nucleoplasmic foci might correspond to telomeres. To investigate this possibility, in the first approach, HeLa S phase cells were hybridized with the hTR-specific oligonucleotide mix and another fluorescently labeled oligonucleotide probe complementary to the G strand of human telomeric DNA. The staining patterns of three representative cells are shown in Figure 2A. In the great majority of nuclei, our telomere-specific oligonucleotide probe specifically stained ~2–10 small nucleoplasmic dotlike foci, suggesting that only a small fraction of HeLa telomeres are accessible for oligonucleotide FISH under the experimental conditions used to detect hTR (see below). Nevertheless, the nucleoplasmic foci stained by the telomere-specific probe frequently reacted also with the hTR-specific oligonucleotide mix (white arrowheads). Even more tellingly, all small nucleoplasmic foci highlighted by the hTR-specific probe, but different from CBs (see also Figures 5 and 6), also reacted with the telomere-specific fluorescent oligonucleotide, lending strong support to the idea that in HeLa S phase cells hTR accumulates at a few telomeres.

To unambiguously demonstrate that the nucleoplasmic foci stained with the telomere-specific fluorescent oligonucleotide correspond to telomeres, GFP-tagged versions of the human telomeric repeat-binding factors, TRF1 and TRF2 (Chong *et al.*, 1995; Bilaud *et al.*, 1997; Broccoli *et al.*, 1997), were transiently expressed in synchronized HeLa S phase cells (Figure 2B). As expected for authentic telomere-specific proteins (Ludérus *et al.*, 1996; Molenaar *et al.*, 2003), both GFP-TRF1 and GFP-TRF2 accumulated in numerous small nucleoplasmic spots. Faithful localization of the GFP-tagged TRF1 and TRF2 proteins was corroborated by the observation that the GFP-TRF1 protein and another fluorescently labeled version of the TRF2 protein, HcRed-TRF2, showed a largely overlapping localization pattern when coexpressed in HeLa cells (bottom panels). When HeLa S phase cells

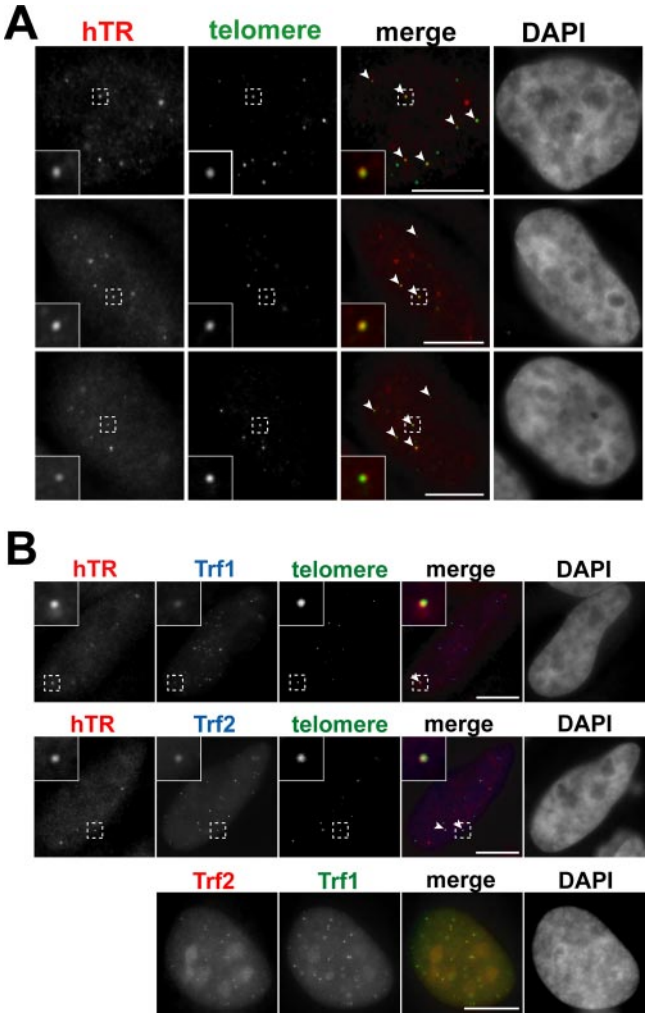

Figure 2. HeLa hTR accumulates at telomeres. (A) Colocalization of hTR with telomeres. HeLa S phase cells were fixed and hybridized with a mixture of hTR- (red) and telomeric DNA-specific (green) fluorescent oligonucleotide probes. Nucleoplasmic foci stained by both hTR and telomeric DNA probes are indicated by arrowheads. (B) Colocalization of telomeres (green) and hTR (red) with ectopically expressed GFP-tagged TRF1 and TRF2 (blue) telomere-specific proteins. Arrowheads indicate nuclear foci containing telomeric DNA, hTR, and GFP-TRF1 or GFP-TRF2. Bottom panels show the distribution of transiently expressed GFP-TRF1 (green) and HcRed-TRF2 (red) proteins. Insets, close-ups of boxed area. Bars, 10 μm.

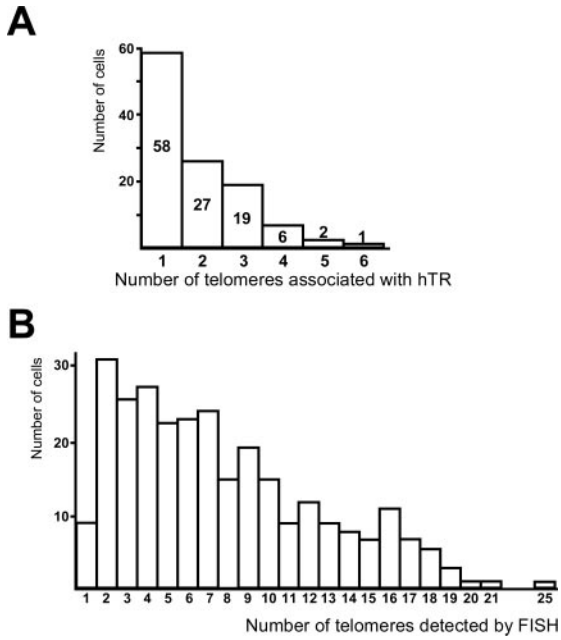

Figure 3. Statistical analysis of accumulation of hTR at telomeres in HeLa S phase cells. (A) Distribution of the number of telomeres accumulating hTR. On inspection of 500 randomly selected S phase cells, colocalization of hTR with telomeres was observed in 113 cells. (B) Distribution of the number of telomeres detected by fluorescent oligonucleotide hybridization performed without chromatin denaturation. On inspection of 350 S phase cells, telomeres were detected in 297 cells.

expressing either GFP-TRF1 (top panels) or GFP-TRF2 (middle panels) were hybridized with a mixture of telomere- and hTR-specific fluorescent oligonucleotides, it became apparent that the GFP-TRF1 and GFP-TRF2 telomere marker proteins also concentrated at the nucleoplasmic foci highlighted by the telomere-specific oligonucleotide probe, demonstrating that they represent a subset of HeLa telomeres. Likewise, the novel hTR-specific nucleoplasmic foci that were successfully stained with the telomere-specific oligonucleotide probe also concentrated the GFP-tagged TRF1 and TRF2 telomeric marker proteins (white arrowheads). Taken together, these results clearly demonstrate that in HeLa S phase cells, hTR specifically accumulates at a few telomeres.

To further substantiate the notion that hTR accumulates at telomeres in a cell cycle-dependent manner, we performed an extensive statistical analysis of synchronized HeLa cells expressing GFP-TRF2 and hybridized with the hTR-specific oligonucleotide probe. On rigorous scrutiny of 500 randomly selected cells representing each cell cycle phase, specific colocalization of hTR with GFP-TRF2-stained telomeres was observed in 113 S (22%), 9 G2 (2%), and 4 G1 (1%) phase cells, indicating that hTR accumulates at telomeres predominantly, if not exclusively, in S phase cells. Indeed, given that in our experiments the efficiency of cell cycle synchronization was ~80% (see Figure 1), the few cells accumulating hTR at telomeres in the G1 and G2 cell populations might represent S phase cells that escaped synchronization. Likewise, considering that in the analyzed S phase cell population ~20% of the cells were in fact G1 and G2 cells, we can estimate that more than 25% of the actual S phase cells accumulate hTR at telomeres at the time of fixation. Normally, hTR accumulation was detected at one or two telomeres with a maximum number of six observed in one cell (Figure 3A). In summary, we concluded that in human HeLa cells, hTR accumulates at telomeres in a cell cycle-dependent manner.

Telomeres Accumulating hTR Possess a Distinct Structure

The finding that a small fraction of HeLa telomeres, actually those ones that are able to recruit hTR, are accessible for in situ oligonucleotide hybridization was rather surprising (Figure 2). Our in situ oligonucleotide hybridization reactions were carried out without heat denaturation in 40% formamide at 37°C and they were not supposed to denature chromatin or tightly packed telomeres. Therefore, our oligonucleotide probe was not expected to interact with telomeric DNA (Moyzis *et al.*, 1987; Lansdorp *et al.*, 1996). Consistent

with this notion, the majority of HeLa telomeres were not detectable in our oligonucleotide FISH experiments, suggesting that those telomeres that are accessible for oligonucleotide hybridization represent a structurally distinct subset of HeLa telomeres. After analysis of 350 randomly scored HeLa S phase cells that had been double-stained with the telomere- and hTR-specific oligonucleotide probes, telomeres were detected in 297 cells (85%). Usually, the number of telomeres visualized by FISH was between 2 and 10 per nucleus, with a maximum number of 25 observed in one cell (Figure 3B). In great accordance with the results obtained above with S phase cells expressing GFP-TRF2, hTR accumulation at telomeres was detected in 75 cells (21%). It is important to emphasize again that hTR accumulation was detected exclusively at those telomeres that were also stained by the telomere-specific fluorescent oligonucleotide probe, raising the fascinating possibility that a structural rearrangement of telomeres is a prerequisite for hTR recruitment.

To learn about the length of telomeric DNA accessible for oligonucleotide FISH, we determined the number of the fluorescently labeled oligonucleotide probe molecules accumulating at telomeres (Figure 4A). To achieve this, we measured the fluorescent light emitted by our probe molecules interacting with 25 distinct telomere foci observed in a HeLa S phase cell. Quantification of the light emitted by known numbers of probe molecules enabled us to define the number of probe molecules interacting with each telomere detected by FISH (Femino *et al.*, 1998). We estimated that the numbers of the telomere-specific oligonucleotide probe molecules accumulating at the least and most intensively stained telomeres ranged from 28 to 340. Given that the utilized oligonucleotide probe was 27-nt-long and assuming that HeLa telomeres are not clustered in the nucleus (Ludérus *et al.*, 1996; Nagele *et al.*, 2001; Molenaar *et al.*, 2003), the fluorescent probe was predicted to cover ~800–9000-nt-long fragments of the telomeric DNA. Because in the test HeLa cell line, the length of telomeres varied between 7 and 20 kb (our unpublished data), we concluded that great portions of the telomeres visualized by FISH were accessible for oligonucleotide hybridization. These results excluded the formal possibility that our G strand-specific oligonucleotide probe hybridized solely to the single-stranded 3′ overhang of the telomeric DNA.

Next, we investigated whether telomeres accessible for in situ hybridization are engaged in DNA replication (Figure 4B). Synchronized HeLa S phase cells hybridized with our telomere-specific probe were counterstained with antibodies directed against the 14- or 34-kDa subunits of the replication protein A (RPA), a single-stranded DNA-binding protein complex that plays a crucial role in DNA replication (Iftode *et al.*, 1999) and the proliferating cell nuclear antigen (PCNA) protein, the auxiliary protein (cofactor) for DNA polymerase delta (Suzuka *et al.*, 1989). RPA14, RPA34, and PCNA showed a very similar, sprinkled nucleoplasmic distribution pattern, but they failed to concentrate at telomeres visualized by FISH, suggesting that the observed structural rearrangement of a fraction of HeLa S phase telomeres is not linked to telomeric DNA replication. Consistent with this conclusion, extension of our analysis to HeLa G1 and G2 phase cells revealed that telomeres highly accessible for oligonucleotide FISH are present also in G1 and G2 cells with a comparable frequency observed for S phase cells (unpublished data).

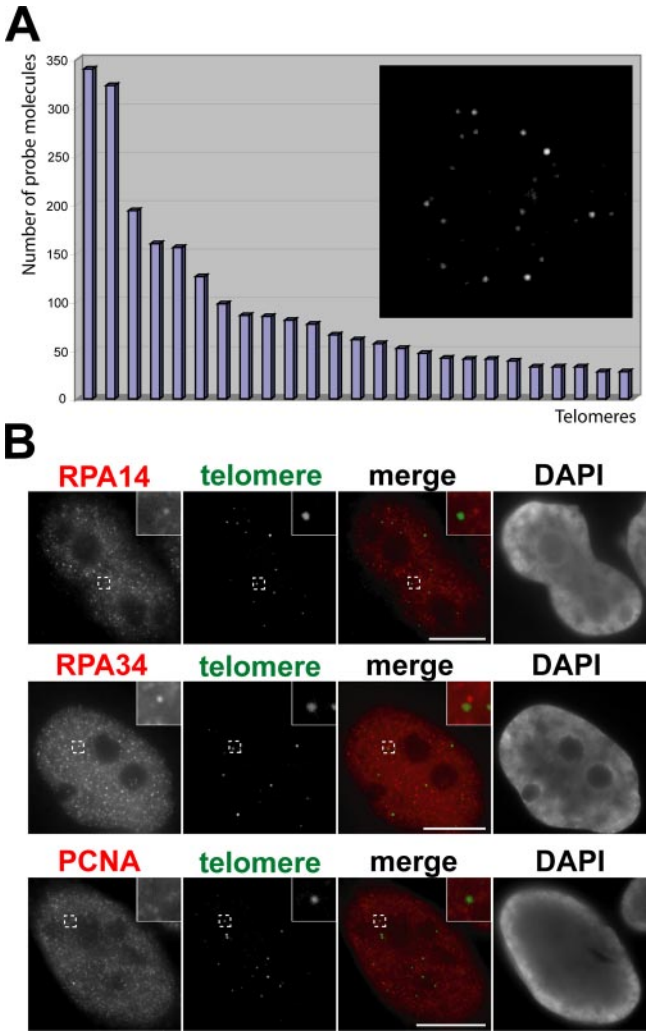

Figure 4. Characterization of HeLa telomeres detected by FISH. (A) Determination of the local quantity of the fluorescently labeled telomere-specific oligonucleotide probe upon FISH analysis of a HeLa S phase cell. The light emitted by hybridizing probe molecules was quantified in order to determine the number of hybridizing oligonucleotides for each detected telomere. (B) HeLa RPA14, RPA34, and PCNA proteins fail to localize to telomeres accessible for in situ oligonucleotide hybridization. HeLa S phase cells were hybridized with the telomere-specific fluorescent oligonucleotide probe and counterstained with antibodies against RPA14, RPA34, and PCNA. Insets, close-ups of boxed area. Bars, 10 μm.

Telomeres Accumulating hTR Frequently Colocalize with CBs

During inspection of HeLa S phase cells hybridized with the telomere- and hTR-specific fluorescent oligonucleotides and counterstained with anti-coilin antibody, we noticed that telomeres accumulating hTR frequently colocalized with CBs (Figure 5, yellow arrowheads). Examination of 93 randomly scored telomeres accumulating hTR revealed that 26% of them (24) were associated with a CB. The fluorescent signals corresponding to telomeres and CBs either completely overlapped or at least, contacted on the periphery, suggesting that there is a direct physical interaction between these structures. It is very important to emphasize that we failed to detect CBs colocalizing with telomeres lacking hTR (Figure 5 and unpublished data). In other words, CBs

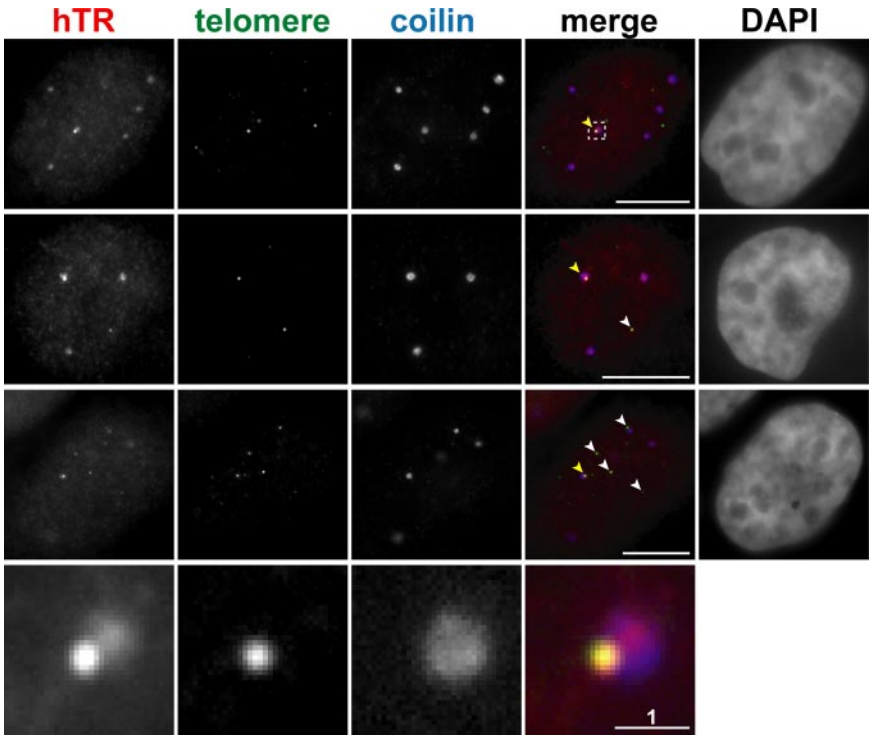

Figure 5. CBs frequently associate with telomeres accumulating hTR. Synchronized HeLa S phase cells were hybridized with hTR- (red) and telomere-specific (green) fluorescent oligonucleotides and counterstained with an antibody directed against coilin (blue). Arrowheads indicate telomeres accumulating hTR. Yellow arrowheads point to telomeres colocalizing with a CB. Bottom panels, a magnified view of colocalization of a telomere and a CB. Unless indicated otherwise, bars, 10 μm.

showed colocalization exclusively with those telomeres that were visualized by oligonucleotide FISH and also concentrated hTR. Finally, telomeres associated with CBs always concentrated high amounts of hTR, whereas in the associated CB, the hTR-specific signal was less intense (see Figure 5, bottom panels).

Next, we examined the formal possibility that the frequent colocalization of telomeres accumulating hTR with CBs was a result of random nucleoplasmic distribution of these structures. To calculate the probability of random colocalization of hTR-containing telomeres and CBs, we applied the locus association test developed for HeLa cells (Jacobs *et al.*, 1999). We calculated that under our experimental conditions, the expected colocalization frequency of hTR-containing telomeres and CBs would be 4.7%. To test whether the difference between the experimental (26%) and expected (4.7%) colocalization frequencies were significant, we compared these values in a χ^2 test. The obtained χ^2 value (92.5) indicated that the probability that the experienced colocalizations of telomeres and CBs were due to random distribution was $<0.01\%$ ($p < 0.0001$). We therefore propose that the observed preference for colocalization of CBs with telomeres accumulating hTR might reflect a functional interaction of CBs and telomeres in HeLa S phase cells.

To confirm the generality of the observed S phase-dependent colocalization of CBs with telomeres accumulating hTR, we investigated the localization of hTR in synchronized human Hep2 laryngeal carcinoma cells (Figure 6). Hep2 cells were hybridized with the hTR- and telomere-specific oligonucleotide probes and immunostained with anti-coilin antibody. As expected, hTR showed a CB-specific accumulation in G1, G2 (unpublished data), and S phase Hep2 cells (Figure 6, top panels). More importantly, in S phase cells, hTR also accumulated at telomeres (middle panels, white arrowheads), which often colocalized with a CB (bottom panels, yellow arrowheads). We therefore concluded that in both HeLa and Hep2 cells, a subset of telomeres are accessible for oligonucleotide FISH and during S phase, these "open" telomeres frequently accumulate hTR and colocalize with CBs.

Dynamic Association of CBs and Telomeres in Living HeLa Cells

We next attempted to confirm the biological significance of the observed colocalization of telomeres and CBs in fixed cells by investigating the intranuclear movements of these structures in living HeLa cells. We also hoped that these in vivo imaging experiments would provide us with some insights into the dynamics of telomere and CB association. To visualize CBs, we utilized a fusion protein of the red fluorescent protein DsRFP and the CB marker protein coilin. As a telomere marker, the GFP-TRF2 fusion protein was used (see Figure 2B). DsRFP-coilin and GFP-TRF2 were transiently coexpressed in synchronized HeLa cells and the intranuclear movements of CBs accumulating DsRFP-coilin and telomeres concentrating GFP-TRF2 were monitored by three-dimensional time-lapse fluorescence microscopy by taking images of the cotransfected S phase cells at 3–4-min intervals over periods of 1–2 h. Previous studies revealed that in live cells CBs either move in the interchromatin space by passive diffusion determined by chromatin dynamics or they are closely associated with chromatin. Finally, a minor, highly mobile fraction of CBs can translocate large distances through the nucleoplasm (Platani *et al.*, 2002; Gorisch *et al.*, 2004).

Transient interaction of fluorescently labeled CBs with telomeres was documented in the nuclei of 47 S phase cells. We found that in 72% of the observed S phase cells, CBs associated with at least one telomere. The measured frequency of CB and telomere association was 1.8 association/h/cell. As a representative example, the fluorescent images of three cells taken at 18 time points is shown in Figure 7. In the nuclei of these cells, five CBs (red) indicated by colored arrowheads were found to transiently associate with telo-

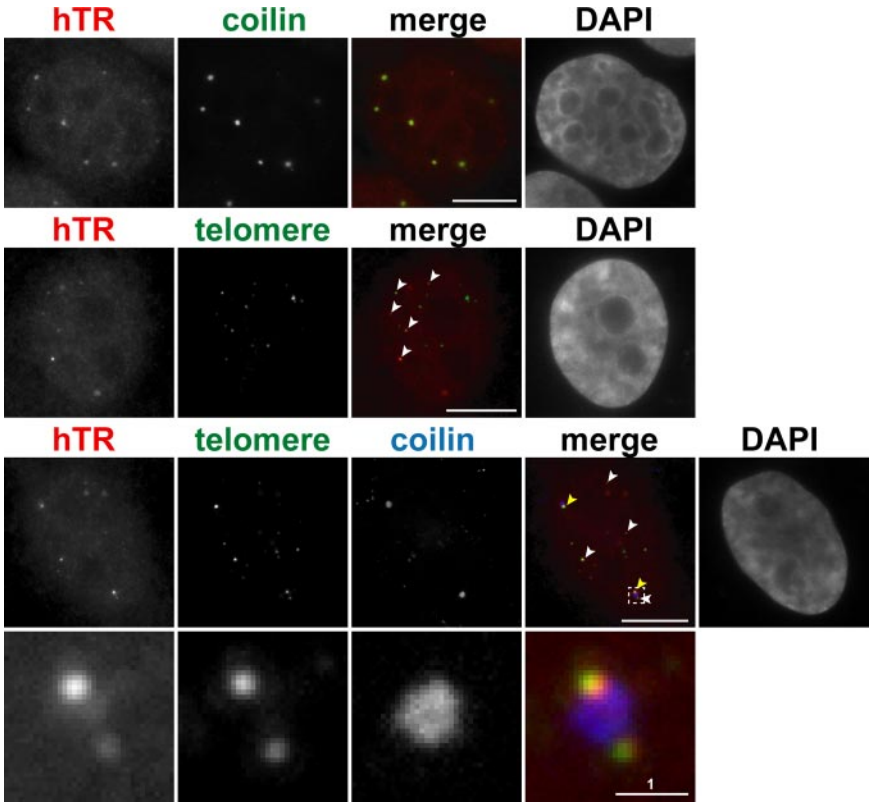

Figure 6. Colocalization of CBs with telomeres accumulating hTR in human Hep2 cells. Hep2 S phase cells were hybridized with hTR- and telomere-specific oligonucleotide probes and counterstained with anti-coilin antibody. An enhanced magnification of the boxed area is shown. Other details are identical to Figure 5.

meres (green). We also observed CBs that rapidly moved through the nucleoplasm before associating with a telomere (an example is indicated by a white arrow in the magnified images). The magnified time-lapse images of associated CBs and telomeres revealed that the two associated structures, although are continuously turning around each other, show no significant translocation movements. Consistent with the notion that the majority of human telomeres are associated with the nuclear matrix (de Lange, 1992; Ludérus *et al.*, 1996; Weipoltshammer *et al.*, 1999), HeLa S phase telomeres showed more constrained intranuclear movements than CBs, but as it has been reported before (Molenaar *et al.*, 2003), translocation of telomeres was also apparent in a few instances (unpublished data). Our in vivo imaging experiments also demonstrated that association of CBs and telomeres is a reversible process. The shortest and longest associations observed between a CB and a telomere lasted for 6 and 75 min, but most frequently, their in vivo colocalization persisted for 12–40 min (Figure 8). After dissociation, CBs and telomeres appeared again as distinct structures, demonstrating that CBs and telomeres have the ability to temporarily associate with each other in HeLa S phase cells.

DISCUSSION

Contrary to its well-established role in telomeric DNA synthesis, the telomerase enzyme has not yet been observed to accumulate at telomeres. In this study, we have systematically investigated the intranuclear localization of the RNA component of human telomerase in synchronized HeLa and Hep2 cells by using oligonucleotide FISH. Our experiments confirmed that in interphase cells, hTR localizes to CBs (Jády *et al.*, 2004; Zhu *et al.*, 2004), but they also revealed that in ~25% of S phase cells, a fraction of hTR accumulates at one or at a few (maximum 6) telomeres that also concentrate the GFP-tagged TRF2 and TRF1 telomere marker proteins and interact with a fluorescent oligonucleotide probe complementary to the G strand of human telomeric DNA. A systematic analysis of HeLa G1, G2, and S phase cells confirmed that accumulation of hTR at telomeres is a cell cycle-regulated process; it occurs predominantly or rather exclusively during S phase when telomere synthesis is known to occur and telomerase shows the highest activity in cancer cells (Zhu *et al.*, 1996).

Because the only known function of hTR is in telomeric DNA synthesis, it seems logical to assume that in our FISH experiments, the observed accumulation of hTR at telomeres reflects telomere elongation events. The finding that in HeLa S phase cells hTR concentrates at only a few telomeres indicates that human telomerase does not act on every telomere in each cell cycle. At the moment, it is difficult to estimate the accurate number of HeLa telomeres recruiting hTR within one cell cycle, because our FISH experiments were unable to determine how long hTR stays associated with telomeres in living cells. However, on the basis of the observed low frequency of hTR accumulation at telomeres, we can assume that only a very small fraction of telomeres are elongated within one cell cycle (see below), provided that HeLa telomeres are not clustered in S phase cells (Ludérus *et al.*, 1996). This hypothesis becomes more appealing in the light of a recent discovery of the Lingner laboratory (Teixeira *et al.*, 2004). By using an elegant genetic approach, Teixeira *et al.* demonstrated that the yeast *Saccharomyces cerevisiae* telomerase extends only a subset of telomeres within one cell cycle.

The notion that in both mammalian and yeast cells, only a fraction of telomeres are elongated in one cell cycle underlines the previous concept that controlling the recruitment of

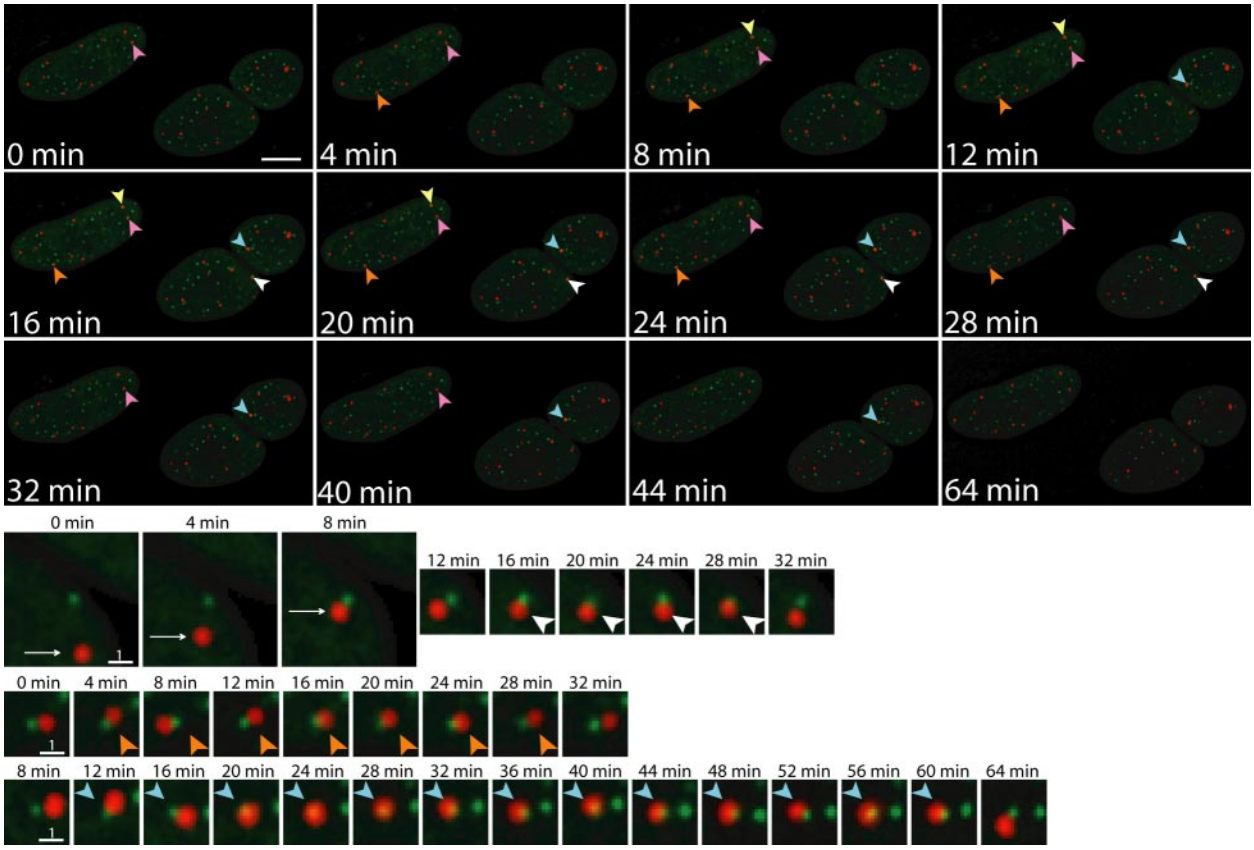

Figure 7. Transient association of CBs and telomeres in living HeLa S phase cells. In vivo time-lapse imaging of HeLa S phase cells expressing DsRFP-coilin and GFP-TRF2 fusion proteins. CBs transiently associated with telomeres are indicated by colored arrowheads. The magnified images of CBs labeled by white, orange, and blue arrowheads and the associated telomeres are shown. White arrow indicates a CB rapidly translocating through the nucleoplasm toward a telomere. Elapsed time is indicated in each image.

hTR to a specific subpopulation of telomeres plays an important regulatory role in telomere synthesis. Earlier works have indicated that the enzymatic properties of telomerase and/or its recruitment to telomeres are controlled by the telomeric chromatin (reviewed in Smogorzewska and de Lange, 2004). The yeast telomerase has strong preference for the shortest telomeres which are open for telomere extension (Teixeira *et al.*, 2004). In contrast, the long telomeres tend to switch to a nonextendible "closed" state. The potential similarity of the yeast and HeLa telomere length regulation mechanisms was emphasized by the unexpected finding of this work that HeLa telomeres fall into two distinct groups based on their accessibility for in situ oligonucleotide hybridization. As expected, the majority of HeLa telomeres failed to interact with telomere-specific oligonucleotide probes in FISH reactions performed without prior heat denaturation (Moyzis *et al.*, 1987; Lansdorp *et al.*, 1996). A few telomeres, usually 2–10 (Figure 3B), however, showed high accessibility for oligonucleotide probes, indicating that they belong to a structurally distinct subset of HeLa telomeres (Figures 2, 4, 5 and 6, and our unpublished data). Importantly, hTR accumulation was observed exclusively at the latter minor class of telomeres which are open for oligonucleotide hybridization. Our observations therefore suggest that similarly to the yeast system (Teixeira *et al.*, 2004), there might exist a binary regulatory switch that controls the telomerase-extendible and -nonextendible states of human telomeres. Mammalian telomerases have been proposed to preferentially elongate short telomeres (Zhu *et al.*, 1998; Ouellette *et al.*, 2000; Hemann *et al.*, 2001; Samper *et al.*, 2001). Quantification of probe oligonucleotides accumulating at HeLa open telomeres suggested that ~1–9-kb fragments of these telomeres are available for in situ hybridization. However, it is difficult to draw firm conclusions from these observations, because some open telomeres may be clustered in HeLa nuclei and the accumulation level of the fluorescent oligonucleotide probe is not necessarily proportional to telomere length because of the potential variability

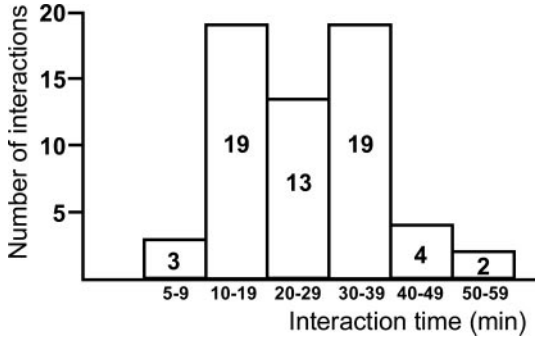

Figure 8. Distribution of the association time of CBs and telomeres in live HeLa cells. Only those interactions that were both formed and terminated during time-lapse imaging were considered.

of the access of the probe to different telomeres. A simple explanation of the elevated oligonucleotide FISH reactivity of open telomeres would be that they undergo DNA replication (Diede and Gottschling, 1999). However, the observation that prominent components of the DNA replication machinery are not detectable at telomeres visualized by FISH and that accessible open telomeres are also present in G1 and G2 nuclei strongly argue against this possibility (Figure 4B). In the future, further studies are required to understand the structural bases of the increased FISH reactivity of HeLa telomeres competent in hTR accumulation.

Although we can postulate with great certainty that hTR accumulating at telomeres functions in telomeric DNA synthesis, thus far no evidence supports hTERT accumulation at telomeres. Unfortunately, in situ localization of endogenous hTERT is very difficult, because of its low cellular concentration and the lack of reliable antibodies. In HeLa cells, transiently overexpressed GFP-tagged hTERT accumulates in the nucleolus or in the nucleoplasm and perhaps also in CBs, but shows no apparent concentration at telomeres (Wong *et al.,* 2002; Yang *et al.,* 2002; Zhu *et al.,* 2004; our unpublished data). In this context, it is noteworthy that telomerase is a processive enzyme that can add multiple copies of the telomeric DNA repeats after a single binding event (Collins and Mitchell, 2002; Cong *et al.,* 2002). In principle, one telomerase particle possesses the capacity to efficiently elongate a telomeric DNA end. This raises the question whether all hTR molecules accumulating at HeLa telomeres are complexed with hTERT or hTR has another not yet identified, hTERT-independent role in telomere biogenesis that necessitates its high local concentration.

The biological importance of accumulation of hTR in CBs has remained conjectural. hTR may be sequestered into CBs to reduce the risk of inappropriate telomere addition to replicating chromosomal DNA. CBs may also function in the biogenesis of telomerase holoenzyme. Hypermethylation of the primary 7-monomethylated guanosine cap of hTR to 2,2,7-trimethylated guanosine and/or assembly of hTR with telomerase RNP proteins may also take place in CBs (Jády *et al.,* 2004; Zhu *et al.,* 2004). In this study, demonstration that 26% of HeLa telomeres accumulating hTR colocalize with CBs raised the intriguing possibility that CBs might have a direct function in telomere biogenesis. According to the most obvious scenario, CBs may deliver hTR to open telomeres that are competent in hTR accumulation and are prone to telomeric DNA elongation. Several observations seem to support this hypothesis. Systematic FISH analysis of HeLa S phase cells revealed that CBs colocalize only with those telomeres that also accumulate hTR and are readily stained with the telomere-specific fluorescent oligonucleotide probe (Figures 5 and 6). In the associated CB-telomere structure, hTR shows a highly asymmetric distribution, it concentrates predominantly at the telomere and it is almost absent from the CB (Figure 5). In general, telomeres associated with CBs contain more hTR than do free telomeres. Finally, in vivo imaging confirmed that in HeLa S phase cells, CBs transiently and specifically associate with telomeres (Figure 7). Because in HeLa S phase nuclei, the frequency of CB association with telomeres is ~1.8 association/h, during the entire S phase (~3–4 h), CBs can associate with 5–7 telomeres. If we accept the hypothesis that CB delivers hTR to individual telomeres, this implicates that no more than 5–7 telomeres are elongated within one cell cycle. Finally, we have to emphasize that CBs stay associated with telomeres for 10–40 min. Therefore, it is possible that CBs also function in other aspects of telomere maintenance; they may carry telomere-specific proteins, may facilitate structural rearrangements of the telomeric chromatin or even may protect telomeres during elongation.

A few human tumor-derived cell lines lacking telomerase activity maintain their telomere length by a recombination-based (alternative lengthening of telomeres [ALT]) mechanism (Lundblad and Blackburn, 1993; Bryan *et al.,* 1995). An emblematic feature of ALT cells is that they carry promyelocytic leukemia (PML) nuclear bodies which colocalize with telomeres (Yeager *et al.,* 1999). The ALT-associated PML bodies also contain the TRF1 and TRF2 telomere-binding proteins and accumulate protein factors involved in DNA synthesis (replication factor A), double-stranded breaks repair, and/or homologous recombination (RAD51 and RAD52 and the RAD50/MRE11/NBS1 complex), leading to the concept that telomere length maintenance in ALT cells occurs at PML bodies (Yeager *et al.,* 1999; Wu *et al.,* 2000, 2003). In vivo imaging of intranuclear movements of telomeric DNA repeats in human U2OS ALT cells, besides confirming that PML bodies associate with telomeres in a dynamic manner, also demonstrated that CBs do not interact with ALT telomeres (Molenaar *et al.,* 2003). Therefore, it seems that association of CBs and telomeres is linked to those proliferating cells in which telomere length is stabilized by telomerase activity. Association of PML bodies with telomeres is not detectable in HeLa cells (Molenaar *et al.,* 2003), lending further support to the hypothesis that CBs play a crucial role in telomere maintenance in telomerase-positive cells, whereas PML bodies function in ALT cell telomere lengthening.

In summary, we demonstrated that in the nuclei of human HeLa and Hep2 interphase cells, a minor subset of telomeres show high accessibility in oligonucleotide FISH. We demonstrated that in S phase cells, the FISH reactive open telomeres can specifically recruit hTR. We showed that 26% of the S phase telomeres accumulating hTR colocalize with CBs. Finally, consistent with the later observation, we demonstrated that in HeLa S phase live cells, CBs transiently associate with telomeres. These surprising observations inevitably led to the provocative hypothesis that CBs may deliver hTR to a structurally distinct subset of HeLa telomeres and/or may function in some aspects of mammalian telomere biogenesis. Testing this fascinating model will be an exciting and challenging task for the future.

ACKNOWLEDGMENTS

We are grateful to Drs. J. Campisi (Lawrence Berkeley National Laboratory, Life Sciences Division, Berkeley, CA) and T. de Lange (Laboratory for Cell Biology and Genetics, Rockefeller University, New York, NY) for providing us with plasmids and Dr. Angus I. Lamond (University of Dundee, Dundee, United Kingdom) for supplying us with anti-coilin antibody. We thank Dr. P. Travo (RIO Integrated Imaging Facility, Montpellier, France) for helpful discussions and technical advice. This work was supported by la Ligue Nationale contre le Cancer.

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